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13. ABSTRACT *(Maximum 200*

The overall goal of this research is to gather detailed information about the cellular distribution and activities of components of the uPA-system in relation to scavenger receptors and RAP in normal and breast cancer cells. We have especially noted a number of differences between the cell lines in either expression levels of these proteins or their localization during the first year of our funding. Scavenger receptors show higher expression levels in estrogen-insensitive compared to estrogen-sensitive and normal cells, whereas newly obtained results for localization of uPAR show that this receptor unlike other GPI-linked proteins was not found in caveolae. Immunocytochemical and immunochemical studies provided evidence that at least one scavenger receptor, LRP, is colocalized with uPAR at the cell surface in several cell types and that both receptors can be coprecipitated. We also obtained evidence that uPAR and uPA are taken up into the cell by endocytosis via clathrin-coated vesicles. We have documented several abnormalities in the very aggressive estrogen-insensitive breast cancer cell line MDA-MB-231--i.e. they have a defect in recycling of LRP and they express a truncated form of uPAR incapable of binding uPA. The absence of the binding site for uPA may have significant impact in uPA cell surface activities and clearance in these cell and might contribute to the cancerous phenotype of these cells.

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FOREWORD

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PROGRESS REPORT

A. STATEMENT OF COLLABORATION

Work funded by this fellowship was done in the laboratory of Dr. Marilyn G. Farquhar. At the beginning of my funding period Dr. Farquhar also received funding through the U.S. Army Breast Cancer Research Program in the form of a Research grant (#DAMD-96-1-6317). Since my fellowship only pays for my salary support, the expenses of my research were covered by Dr. Farquhar's grant. Thus there is a partial overlap between my Specific Aim (SA) #1 and the SAs #1 and #2 in Dr. Farquhar's proposal in regard to the subcellular localization of scavenger receptors, urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), plasminogen activator inhibitor (PAI-1), and the receptor-associated protein (RAP). Dr. Kuemmel, a postdoctoral fellow working in Dr. Farquhar's laboratory, carried out the immunocytochemical studies, and I concentrated on the immunochemical aspects of our proposals during this second year of funding. Ms. Popa, a graduate student in our laboratory was involved in the preparation and initial characterization of anti-human megalin antibodies and in the purification of human alpha-2 macroglobulin. During the second year of our funding, she also participated in the cloning of human uPA and human PAI-1. Due to my experience and knowledge of the established immunochemical and immunocytochemical techniques in Dr. Farquhar's laboratory I was substantially involved in guiding Dr. Kuemmel and Ms. Popa through these protocols and in designing experiments necessary for their work on breast cancer cells.

This decision to combine our effort increased our productivity and, as this report and Dr. Farquhar's report will clearly document, generated a significant amount of novel insight on the role of scavenger receptors in breast cancer cells and in particular about their role in the uPA-system.

B. BODY OF WORK

I. Preparation and characterization of polyclonal antibodies against human megalin and characterization of new monoclonal antibodies against human LRP and human uPAR.

In the first year of our funding period we evaluated the potential of our antibodies, made against human uPAR and human LDL-receptor-related protein, LRP (1), to detect their respected antigens in a variety of breast cancer cells. The initial preparation of a new anti-human megalin antibody was time consuming and led to the present situation where we have now characterized this antibody in breast cancer cells.

Polyclonal antibody (PAb) sera against the ectodomain of human LRP (1) and against the cytoplasmic domain of human LRP (1) were previously available in our laboratory as well as a polyclonal antiserum generated against a recombinant human uPAR made in bacteria. The design of our experiments performed during the second year of our funding required some new monoclonal antibodies (MAb). It became evident that the PAb against the ectodomain of LRP was not useful to study the surface expression of human LRP in mammary epithelial cells. Therefore, we received MAb against the ectodomain of human LRP as a generous gift from Dr. D. Strickland (American Red Cross, Bethesda, Maryland). Furthermore, we received MAb against the cytoplasmic domain of human LRP and an N-terminal domain of human uPAR as generous gifts from Dr. J. Herz (University of Texas Health Science Center, Dallas, Texas) and Dr. M. Shuman (UC San Francisco), respectively. In addition, we purchased anti-uPAR (human) MAb and anti-uPA MAb from American Diagnostica Inc., and MAb against human uPA and human PAI-1 from Calbiochem.

We have provided a detailed list of all our antibodies used in these studies and have indicated in which assay they are most useful (see TABLE I).

II. INTRODUCTION

Proteases are of special importance in the pathogenesis of breast cancer because they play a key role in invasion and metastasis which requires the action of tumor-associated proteases to disrupt the tumor matix. Specifically, tissue concentrations of the urokinase-type plasminogen activator, uPA, its plasma membrane receptor, uPAR, and plasminogen-activator-inhibitor (PAI)-1 have been shown to have prognostic value in relationship to the progression of breast cancer (2, 3). Increased activity of uPA has been linked to cell migration and invasion during embryogenic development as well as to invasive growth and metastasis (2). Overexpression of uPA, its receptor, uPAR, and the uPA inhibitor, PAI-1, in primary tumors could be linked to an increased metastatic potential of these tumor cells.

Recent studies have reported that significantly higher levels of uPA and PAI:1 are found in mammary carcinomas than in their normal mammary epithelial cell counterparts (4-10) indicating a disturbance of the very delicate balance between activation and inactivation of uPA in tumor cells. It is known that this increase in tumor-associated proteases promotes invasion and metastasis through the dissolution of the surrounding basement membranes and tumor matrix.

The overall purpose of this work is to define the mechanisms responsible for the increased expression of PAI-1, uPA, and its receptor in breast cancers associated with increased potential for recurrence and metastasis. The working hypothesis to be tested is that the delicate balance between protease activation and inactivation/clearance is upset in breast cancers with high metastatic potential. Recently discovered scavenger receptors, i.e., LRP and megalin (11-13), have been described to bind and to endocytose uPA:PAI-1 complexes (14-19).

Both of these receptors are members of the LDL-receptor gene family, have a very similar overall structure in their extracellular domain and bind similar ligands in in vitro assays on cultured cells. Based on their ability to bind and clear uPA:PAI-1 complexes, these scavenger receptors are thought to play a significant role in biologic and pathologic processes involving tissue remodeling, i.e., embryonic development, wound healing, and malignant cell invasion.

The successful completion of the experiments proposed in the original application will shed light on a number of important questions including: 1) Is their a quantitative difference in the levels of scavenger receptor expression in normal mammary epithelial cells vs. breast cancer cells; 2) What are the trafficking itineraries of scavenger receptors and uPAR in both normal and tumor-derived cells; and 3) Are scavenger receptors involved in the down-regulation of cell surface uPA activities through co-internalization of uPA and uPAR? As a result of these studies we will gain insights as to the mechanisms responsible for the abnormal accumulation of uPA, its receptor and PAI-1 in breast tumors.

III. DETAILS OF PROGRESS

Our working hypothesis was that the increased expression of uPA, uPAR, and PAI-1 in breast cancers with high metastatic potential is due to abnormalities in the clearance of these molecules by scavenger receptors.

Work has been carried out under each specific aim as follows.

1. SPECIFIC AIM #1 (Year 1): Determine the Localization of LRP and Megalin in Normal and Tumor-derived Mammary Epithelial Cell Lines.

Background information from previous work:

Previous work has established that normal human mammary epithelial cells express either megalin or LRP (20, 21). At steady state the majority of the receptors are localized at the cell

surface in clathrin-coated pits, whereas RAP is predominantly found intracellularly and in the rough ER (12, 20, 22-24). Except for our preliminary studies, the expression of scavenger receptors had not been studied in normal or tumor-derived mammary cell lines.

Results listed in our last report established that all the breast cancer cell lines examined express LRP and megalin. We showed that there are differences in the expression and distribution of megalin but not LRP between the normal mammary epithelial cell line and tumor-derived cell lines. Of particular interest is that megalin was only expressed in breast cancer cell lines and was not detectable in normal cells. Our goal was to determine whether there are differences in the expression levels of these proteins between normal and estrogen-sensitive and/or estrogen-insensitive breast cancer cells which could explain the increased levels of components of the uPA-system in tumors. Hypothetically, a decreased expression of scavenger receptors at the cell surface could lead to a accumulation of uPA and PAI-1 extracellular due to decreased clearance and intracellular degradation of these complexes.

Additional Results Obtained in Year 2:

1. Immunocytochemical Localization of Scavenger Receptors:

Methods: The immunocytochemical methods used were the same as those used in our previous studies of the distribution of megalin and LRP in various cell lines (1, 24, 25) and in the initial studies of our funding period. For more detailed information on specifics of method and antibodies see Dr. Farquhar's Progress Report #DAMD-96-1-6317.

Immunofluorescence: In brief, cultured cells were fixed either in paraformaldehyde or in paraformaldehyde-lysine-periodate (PLP) for preparation of semithin (1 μm) cryosection. Incubation with primary antibodies (2 h) was followed by FITC- or Texas Red-conjugated secondary antibodies. Results were obtained using a Zeiss Axiophot equipped for epifluorescence.

Immunogold electronmicroscopy: In brief, cells were fixed in PLP or a mixture of paraformaldehyde/glutaraldehyde or sequentially in different concentrations of paraformaldehyde alone. Cells were cryoprotected by infiltration with sucrose and processed for cryosectioning as described (26, 27). Details of the sequential immunogold staining of the ultrathin cryosections are also given in recently published papers (1, 24, 28, 29).

Results: We localized LRP in MDA-MB-231 cells and Hs578T cells by concentrating on its distribution on the apical cell surface where this receptor is expressed. The results were similar in the two cell lines: LRP is located on the plasma membrane in a punctate (dot-like) distribution. Localization of LRP at the plasma membrane was confirmed at the EM level.

For details see Figures #4, #5, and #6 in Dr. Farquhar's Progress Report #DAMD-96-1-6317.

Summary of Results and Questions Answered: The results of these studies have clearly established that there are differences between a normal mammary epithelial cell line and estrogen-sensitive and estrogen-insensitive tumor-derived cells in the levels of scavenger receptor expression, but not of RAP.

Our overall goal under Specific Aim #1 is to determine whether there are derangements between normal and tumor-derived cells in the expression or location of scavenger receptors that might be responsible for the higher levels of uPA, uPAR, and PAI-1 found in breast cancers. Our findings to date suggest that abnormal accumulation of uPA, uPAR, and PAI-1 cannot be explained by the absence of scavenger receptors since all tumor-derived cells express either LRP or megalin.

2. SPECIFIC AIM #2 (Year 2): Determine the Levels of Expression of LRP and Megalin in Normal and Tumor-derived Mammary Epithelial Cells.

Background information from previous work:

Our findings from the first year of our funding period clearly indicated that the expression of LRP significantly varied between the normal and tumor-derived cells. Total LRP expression in breast cancer cells is highest in estrogen-insensitive MDA-MB-231, which is the most metastatic and invasive cell line tested in our experiments. LRP expression is the lowest in estrogen-sensitive MCF-7, which is the least aggressive cell line tested here. Megalin was not found in the normal cells using immunocytochemistry but was detected in Hs578T cells in immunofluorescence and immunoprecipitation.

Although, immunoprecipitation results on surface expression of LRP in normal mammary cells were not available, the surface expression pattern in breast cancer cells seemed to reflect the relative amounts of this receptor as detected in total cell lysates.

Our goal was to test the other available mammary tumor cell lines for expression of megalin using the new anti-megalin (human) PAb to establish a complete correlation between megalin expression and malignancy of these cells.

Additional Results Obtained in Year 2:

1. The Normal Mammary Epithelial Cell Line, 184-B5 Expresses Megalin:

We used tumor-derived and normal mammary gland epithelial cell line to compare expression levels of megalin and LRP by semiquantitative immunoblotting. Methods were used as described previously (22, 25).

Methods: Cells were homogenized and membrane pellets were obtained by centrifugation at 100,000 x g. Membranes were solubilized with CHAPS detergent, equal amounts of protein (50 μg) were fractionated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with our newly generated anti-human megalin polyclonal antibodies.

For more details see Figure #1 in Dr. Farquhar's Progress Report #DAMD-96-1-6317

Results: At the time of last year's progress report we had only performed preliminary immunofluorescence (IF) studies using our new anti-human megalin antibodies and had not yet performed the immunoblotting survey. Since then we have performed immunoblot analysis on tumor-derived breast cancer cell lines MDA-MB-231 (estrogen-insensitive) and MCF-7 (estrogen-sensitive), the normal mammary epithelial cell line 184-B5, and a new estrogen-insensitive breast cancer cell line, MDA-MB-468. In agreement with the IF data, immunoblotting confirmed the expression of megalin in all tumor-derived cell lines. In addition, with our new high titer anti-megalin antibodies we have also detected megalin in membrane fractions obtained from the normal 184-B5 cells which we had shown to be negative by IF. Based on the immunoblotting results which are more sensitive than IF, we conclude that all cell lines--both normal and breast tumor-derived--surveyed express megalin.

For more details see Figure #1 in Dr. Farquhar's Progress Report #DAMD-96-1-6317.

2. Identification of a Breast Cancer Cell Line that Failes to Express LRP:

Methods: In brief, proteins from confluent cell monolayers were extracted in 10 mM CHAPS and equal amounts of protein were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) Immobilon-P membranes for Western blot analysis. Membranes were incubated with primary antibodies against LRP and processed using an Enhanced Chemiluminescence (ECL) detection kit for semi-quantitative assessment of protein expression.

We used anti-uPAR antibodies as an internal control since we already showed expression of uPAR in MDA-MB-231 and 184-B5 cells in our last report.

Results (see Figure 1): uPAR was expressed in all three cell lines with the highest amount detected in MDA-MB-231 cells. Expression of LRP was similar in MDA-MB-231 and the normal mammary epithelial cell line, 184-B5. Surprisingly, LRP could not be detected in MDA-MB-468 cells.

Conclusion: Taken together, these data show that all breast cancer and the normal cell line express scavenger receptors to various levels. With the exception of MDA-MB-468 cells, all our cell lines express LRP. Since our immunoblotting data are based on equal amount of protein, the negative blotting data for MDA-MB-468 cells could be due to very low expression level for LRP similar to the data on MCF-7 cells listed in our last report. Interestingly, MCF-7 cells (estrogen-sensitive) and MDA-MB-468 cells (estrogen-insensitive) express none or only a very small amount of LRP and both are descibed as the least aggressive (metastatic) tumor-derived cell lines tested here. Whether there is a significance to this correlation between low or no expression of LRP and the lack of high metastatic potential has to be determined.

In addition, MDA-MB-468 cells with none or low expression level of LRP but significant expression of megalin might represent an ideal cell line to investigate the interaction of the uPA-system with megalin in particular. Current data concentrate solely on the role of LRP in the interaction of scavenger receptors with the uPA-system.

3. Cell Surface Expression of LRP

Methods: Confluent cell layers of tumor-derived mammary epithelial cells and a human fibrosarcoma cell line, HT1080, used as a control cell line were surface radioiodinated following the lactoperoxidase method as described in our previous work on scavenger receptors (1). In brief, cells were incubated at 4°C in buffer containing lactoperoxidase (10 U/ml) and $Na^{125}I$ (1.0 mCi). The labeling reaction was started by the addition of 0.1% H_2O_2. Cells were washed and MAb 8G1 against the ectodomain of human LRP were bound to the cells surface at 4°C for 1h. After additional washing at 4°C, proteins were extracted in 10 mM CHAPS and aliquots were processed for immunoprecipitation with protein G-agarose beads at 4°C for 18h. Immunoprecipitated proteins were separated on SDS-PAGE and gels were exposed to film.

Results (see Figure 2): MCF-7 (estrogen-sensitive), MDA-MB-231 and Hs578T (estrogen-insensitive) cells express LRP at the cell surface. MDA-MB-231 cells show the highest amount of LRP. HT1080 cells also express LRP at the cell surface. Interestingly, this LRP could be co-immunoprecipitated with anti-uPAR MAb.

Conclusion: Our data show significant expression levels for LRP at the cell surface of all tested cells. The fact that LRP and uPAR co-immunoprecipitate even in the presence of mild detergent suggests that there is a strong interaction between both receptors at the cell surface of HT1080 cells. This interaction of uPAR and LRP will be further investigated under Specific Aim #4 where we will show additional evidence confirming the presence of uPAR:LRP complexes at the cell surface of tumor-derived cells.

3. SPECIFIC AIM #3 (Year 2): Compare the Expression and Cellular Distribution of PAI-1, uPA, and uPAR in Relation to Scavenger Receptors in Normal Mammary Epithelial Cells vs. Tumor-derived Cell Lines.

The goal of these studies is to determine where in or on cells uPA, uPAR, and PAI-1 are located and whether they colocalize in the same organelles, especially whether they are found in the same microdomains of the cell membrane. By determining the precise subcellular localization of uPA, PAI-1, and uPAR, these studies will help us to better define their functional relationship in breast cancer cells with regard to localized cell surface proteolytic activities.

Background from previous work:

Breast cancer with high metastatic potential show increased expression of PAI-1 (8, 30, 31) and uPA (12, 30, 31) and both could be detected with immunohistochemical methods. In addition, PAI-1 could be located in the extracellular matrix (8). Distribution of uPAR in normal mammary epithelial cells seems to be limited to focal adhesions. However, in MCF-7 cells it has been detected at the leading edge of the migrating cells.

In our last report we initially concentrated on the subcellular localization of uPAR. In our studies we could detect uPAR in normal and tumor-derived cells. Use of antibodies directed against the occupied (uPAR:uPA) and the unoccupied (uPAR) receptor indicated a redistribution of uPAR to the basal membrane of these cells. Staining for uPAR was stronger in estrogen-insensitive than in estrogen-sensitive cell lines. Expression of uPAR could be confirmed by immunoblotting and immunoprecipitation

Immunofluorescence on uPA and PAI-1 were different in estrogen-insensitive tumor-derived cells vs. the normal mammary epithelial cells. The latter showed uPA localization only at the apical surface whereas in tumor-derived cell lines uPA was located at focal adhesions reflecting the same pattern as obtained with antibodies against occupied uPAR.

We planned to further examine these observations using immunoelectron microscopy.

Additional Results Obtained in Year 2:

1. Subcellular Localization of uPAR

Methods: Cells were prepared for immunocytochemistry as described under Specific Aim #1. For more details see legends to Figures #4, #5, and #6 in Dr. Farquhar's Progress Report #DAMD-96-1-6317.

Results: In brief, we localized uPAR in MDA-MB-231 cells and Hs578T cells by concentrating on its distribution on the apical cell surface where this receptor is usually expressed. The results were similar in the two cell lines: uPAR is located on the plasma membrane in a punctate (dot-like) distribution and the localization at the plasma membrane was confirmed at the EM level. Since these data were gathered from double-labeling experiments with LRP (see results for LRP under Specific Aim #1) we can say that the distribution of both receptors partially overlaps and that they are partially colocalized along the plasma membrane.

To obtain evidence on the proposed localization of GPI-anchored proteins in specialized plasma membrane microdomains called caveolae (32) we carried out double labeling for uPAR and caveolin, a well characterized marker for caveolae. To our surprise we found no overlap in the distribution of uPAR and caveolin by either IF or immunogold labeling.

For more details see Figures #4, #5, and #6 in Dr. Farquhar's Progress Report #DAMD-96-1-6317.

Summary of New Results: By immunocytochemistry we have shown that uPAR and LRP demonstrate an overlapping distribution at the cell surface of breast cancer cells suggesting that they are co-internalized following ligand binding. To our surprise, we were unable to find uPAR

in caveolae in tumor-derived breast cancer cells regardless of the fact that it is a GPI-anchored membrane protein.

4. SPECIFIC AIM #4 (Year 3): Determine the Fate of uPA, uPAR, Scavenger Receptors, and uPA:PAI-1 Complexes at the Cell Surface of Normal and Tumor-derived Mammary Epithelial Cells

Background from previous work:

Recently, a model has been proposed where uPA secreted by migrating cells will be activated when bound to uPAR at the cell surface. Activity of uPA is regulated and inactivated by complexing with PAI-1. Furthermore, it was suggested that uPA:PAI-1:uPAR complexes are then cleared from the cell surface via scavenger receptors (33-37). However, in breast cancers with metastatic potential increased expression of uPAR, uPA, and PAI-1 suggesting an abnormality in the binding and/or clearance of the complexes via scavenger receptors.

Our initial studies showed similar binding affinities for alpha-2 macroglobulin (α2M), a specific ligand for LRP, for all tested cell lines. Binding capacities for α2M on these cell lines indicated that estrogen-insensitive and the normal cell line expressed more functional LRP at the cell surface as the estrogen-sensitive cell lines. MDA-MB-231 cells showed 6-17x more functional receptor on their cell surface than the other cell lines. However, uptake and degradation of α2M on MDA-MB-231 cells suggested that LRP undergoes one rapid round of internalization to deliver its bound ligand to lysosomes, and then is incapable of recycling to the cell surface for additional rounds of ligand uptake. This could reflect an abnormality in the mechanism of LRP recycling in this cell line.

Further studies in this specific aim were designed to establish in more detail whether there are abnormalities in LRP's clearance function, i.e., internalization and endocytic trafficking.

Due to the unique mechanism of interaction between uPA and uPAR on one hand and between uPA:PAI-1 complexes and LRP on the other, the ligand binding and ligand uptake experiments as used for studies on LRP had to be adjusted for the studies on uPAR. The most specific ligand for human uPAR is human uPA based on the high species specificity between this protease and its receptor. However, after binding of uPA and in the presence of endogenous PAI-1, uPA:PAI-1 complexes could rapidly form on uPAR and the receptor would immediately release uPA in the form of uPA:PAI-1 complexes. Therefore, uPA is not a suitable ligand for our binding studies on uPAR. Alternatively, we planned to clone and express an aminoterminal fragment (ATF) of human uPA using reverse transcriptase-PCR (RT-PCR) on total RNA prepared from U937 cells. ATF consists only of the binding domain for uPAR and will neither show a proteolytic activity nor possess the binding site for PAI-1. We planned to use radiolabeled ATF to quantitate the expression levels of uPAR at the surface of tumor-derived and normal mammary epithelial cells.

Additional Results Obtained in Year 2:

A. Assessment of Functionality of LRP and uPAR

Initial studies showed that uptake and degradation of α2M in MDA-MB-231 cells does not occur in a linear and time-dependent fashion. Results suggest that internalized LRP is incapable of recycling to the cell surface and could reflect an abnormality in the mechanism of LRP recycling in this cell line.

1. Recycling of LRP is Blocked in MDA-MB-231 cells:

Methods: After cell surface iodination (see Methods to Figure #2) cells were incubated with α2M at 4°C for 1 h followed by incubation at 37°C for 0, 10, 20, 30, and 40 min, respectively. Cells were washed at 4°C and incubated with anti-LRP MAb (8G1) for cell surface immunoprecipitation (see Methods to Figure #2). Precipitated proteins were separated by SDS-PAGE and processed for autoradiography. The amount of radioiodinated LRP at the cell surface at each time point was determined by PhosphorImager analysis. Data are shown as percent of the total radioactivity detectable at the cell surface at 4°C.

Results (see Figure 3): Normal mammary epithelial cells, 184-B5, and a cell line of normal rat kidney cells (NRK) were used as controls. During the first 20 minutes of incubation at 37°C, all three cell lines showed an initial decrease in the radioiodinated surface pool of LRP by 60%. In our control cell lines, after an additional incubation at 37°C, about 80% of the total radioiodinated LRP (expressed at the cell surface at 4°C (= 0 min at 37°C)) could be detected at the cell surface. These results indicate that both control cell lines showed very similar recycling kinetics for LRP. Although, MDA-MB-231 cells show also an initial decrease of surface LRP of 60% during the first 20 min, the detectable amount of radioiodinated LRP at the cell surface remained unchanged over time indicating that the internalized LRP is not recycled.

Conclusion: Our data suggest that in MDA-MB-231 cells LRP undergoes one rapid round of internalization to deliver the pre-bound ligand (α2M) to lysosomes. LRP is incapable of recycling to the cell surface for additional rounds of ligand uptake. Thus we have obtained evidence by two different methods, i.e., degradation studies with α2M and surface IP of the receptor, for a defect in LRP recycling in MDA-MB-231 cells. It will be of further interest to investigate if this abnormality is due to a defect in the LRP molecule itself or due to a defect in the cellular recycling machinery overall.

2. Cultured MDA-MB-231 Cells Express a Truncated Form of uPAR at the Cell Surface:

Methods: For immunoprecipitation analysis, cells were radiolabeled by lactoperoxidase-mediated cell surface iodination as described in Methods to Figure #2, and incubated with 10 Units of phosphoinositol-specific phospholipase C (PI-PLC) at 4°C for 30 min to release uPAR from the cell surface. uPAR was then immunoprecipitated from the PI-PLC released soluble fraction or from residual cell lysates obtained with CHAPS detergent using anti-$uPAR_{MS}$ MAb. Precipitated proteins were separated by SDS-PAGE. In some experiments, proteins were transferred to PVDF membranes for immunoblotting with anti-uPAR PAb to confirm that the immunoprecipitated protein is indeed uPAR.

For the uPA-derived peptide (AE78) binding studies, AE78, which represents the binding site in uPA for uPAR (38), was radioiodinated using the Iodo-Bead method according to the manufacturer's instructions (Pierce). Radiolabeled peptide was separated from free iodine by gel filtration chromatography. MDA-MB-231 cells and control cell line A431 (derived from human epidermoid cancer) were grown in 12-well culture plates to confluency, and incubated with 1 nM, 10 nM, or 100 nM ^{125}I-AE78. Non-bound peptide was removed by rinsing cells and bound peptide was quantitated by gamma counting. Non-specific binding was determined by a parallel co-incubation with a 200-fold molar excess of unlabeled AE78.

For immunocytochemistry, semithin (1 μm) sections were prepared and incubated with three different Domain-specific anti-uPAR MAb. Bound antibodies were detected with FITC-conjugated secondary antibodies. For more detailed information on specifics of method

and antibodies see Methods and legend to Figure # 11 in Dr. Farquhar's Progress Report #DAMD-96-1-6317.

Results (see Figure 4) : In the process of characterizing the cell surface pool of uPAR we discovered that the uPAR at the surface of MDA-MB-231 cells has lower relative molecular mass by SDS-PAGE than in other cell lines including the normal mammary epithelial cell line, 184-B5. This was identified by surface iodinating cells and immunoprecipitating uPAR following its complete release **(A)** from the cell surface with PI-PLC (which specifically cleaves GPI-anchored proteins) **(B).** Immunoblotting of the immunoprecipitated proteins with anti-uPAR antibodies confirmed that the truncated protein is in fact uPAR **(C).**

Results (see Figure 5): To determine if this truncated form of uPAR at the surface of MDA-MB-231 cells is functionally active to bind uPA, we investigated whether these cells are able to specifically bind the uPA-derived peptide AE78. The AE78 peptide represents the amino acid sequence that corresponds to the binding domain of uPA for uPAR (38) and demonstrates a very high affinity for the receptor (Kd=10 nM). Our results show that although A431 cells, which express functional intact uPAR on their surface, are able to specifically bind the radiolabeled peptide, the truncated form of uPAR on MDA-MB-231 cells is unable to bind AE78 These results suggest that the truncated form of uPAR lacks Domain 1 of the receptor which contains the functional binding site for uPA (38, 39).

These results were further confirmed by immunocytochemistry using uPAR domain specific antibodies. For more detail see Figure #11 in Dr. Farquhar's Progress Report #DAMD-96-1-6317. In brief, MDA-MB-231 cells do not stain with uPAR anti-Domain 1 antibodies, but do stain with antibodies which recognize an epitope between Domains 1 and 2 and with antibodies which recognize only Domain 2. These results confirm that the Domain 1 is absent from the truncated form of uPAR.

Conclusion: MDA-MB-231 cells express a smaller form of uPAR at the cell surface than normal mammary gland epithelial cells. In view of published data (40) regarding truncated forms of uPAR expressed at the cell surface of different cells, we suggest that high expression of uPA by MDA-MB-231 cells in culture could lead to a cleavage of uPAR between Domain 1 and 2 by activated receptor-bound uPA. This effect would be emphazised by the low amount of PAI-1 secreted by those cells, which could be detected only in the extracellular matrix of MDA-MB-231 cells (see report last year).

Treatment with N-glycosidase F indicated that the differences in the apparent mobility of uPAR in the gel was not due to differences in N-linked glycosilation (data not shown). O-glycosylation sites could not be described for uPAR based on sequence analysis of the cloned cDNA (41).

To test the hypothesis that the truncation of uPAR at the cell surface of MDA-MB-231 cells is due to enzymatic cleavage and not to an defect during transcription we performed RT-PCR on all cell lines.

Methods: Total RNA was prepared from one confluent 100-mm cell culture dish of each cell line using TRIZOL Reagent according to the manufacturer's instruction (Life Technologies). To clone full-length uPAR and the individual domains, D1-D3, first strand cDNA synthesis was accomplished by using total RNA primed with oligo (dT) and random hexamer oligonucleotides and reverse-transcribed with Superscript RNase H/RT according to the manufacturer's instruction. Subsequent PCR amplification of the different encoding regions (D1-D3) and the full-length uPAR was performed using specific oligonucleotides (100 pmoles/µl). The sequence of all oligonucleotides is available upon request.

Amplification was performed using Pfu polymerase according to the following PCR protocols. For Domains D1-D3: 2 min at 94°C; 3 cycles of 30s at 94°C/ 1min at 40°C/ 1min at 72°C; 30 cycles of 30s at 94°C/ 1min at 55°C/ 1min at 72°C; 7 min at 72°C. Full length uPAR was amplified following the same protocol except the initial set of 3 cycles was eliminated. Total volume of PCR reaction was 100ul and 10% was run on 1.5% agarose gel. As control plasmid we used uPAR-encoding cDNA obtained from the Human Genome Project.

Results (see Figure 6): We found that all cell lines tested -- the normal mammary epithelial cell line (184-B5) and three tumor-derived cell lines (MCF-7, MDA-MB-231, and MDA-MB-468) -- express full length uPAR (**A).** In addition, RT-PCR amplification of all three domains (D1-D3) of uPAR were detected and corresponded to the expected sizes (**B).** These results confirm the results based on immunoblotting that all cell lines tested express full length uPAR that has the expected three domain structure.

Conclusion: Our results clearly demonstrate that the truncation of uPAR detected on the cell surface expressed receptor is not due to an defect on the transcription level in MDA-MB-231 cells.

B. Assessment of Endocytic Uptake of uPAR.

The colocalization of uPAR and LRP as demonstrated in our immunofluorescence and immunogold studies (see Figures #4, #5, and #6 in Dr. Farquhar's Progress Report #DAMD-96-1-6317) suggested that these two receptors may be physically associated in a molecular complex at the cell surface. In addition and most important, data shown here in Figure #2 already suggested that HT1080 cells express a complex of LRP and uPAR at their cell surface. We further explored the possibility that uPAR and LRP could be co-immunoprecipitated together. The human fibrosarcoma cells, HT1080, were used in this procedure to optimize our co-immunoprecipitation conditions since they are well described to express components of the uPA-system (42) and LRP (unpublished observation).

1. Expression of uPA, PAI-1 and uPAR:LRP Complexes in HT1080 cells

Methods: Whole cell lysates (WCL) from HT1080 cells were prepared in 10 mM CHAPS. Proteins were separated by non-reducing SDS-PAGE and transferred onto PVDF membranes for immunoblot with anti-uPAR, anti-uPA, and anti-PAI-1 antibodies. In parallel experiments, HT1080 cells were incubated with anti-LRP MAb (8G1) at 4°C for surface immunoprecipitation (see Methods to Figure #2) and subsequently transferred onto PVDF membranes for immunoblotting.

Results (see Figure 7): A. Immunoblot on WCL under non-reducing conditions confirmed expression of uPA, PAI-1, and uPAR in HT1080 cells. Non-reducing conditions were required since the anti-uPA and anti-PAI-1 antibodies used in these experiments do not blot proteins under reducing conditions. Anti-PAI-1 antibodies recognize two additional high molecular weight bands at 90-100 kDA and 150-160 kDA, respectively. Note, the anti-uPAR antibodies also recognize an additional ptorein band at approximatly 90-100 kDA. **B.** Sequential surface immunoprecipitation (anti-LRP) and immunoblotting with anti-LRP and anti-uPAR antibodies, respectively, clearly showed that uPAR was co-precipitated with LRP.

Conclusion: Taken together, we convincingly demonstrated the co-precipitation of uPAR and LRP using anti-LRP MAb or anti-uPAR MAb (see Figure #2) from the surface of HT1080 cells. These findings indicate that these receptors are complexed at the cell surface of HT1080 cells.

With the proper conditions now worked out we will determine if a uPAR:LRP complex is also present at the surface of breast cancer cells.

Immunoblotting (IB) with anti-PAI-1 MAb under non-reducing conditions showed similar bands than IB with anti-uPAR MAb suggesting that PAI-1 could also be complexed with surface expressed uPAR under our experimental conditions. This has to be further investigated using sequential IP and immunoblotting techniques.

2. Subcellular Fractionation of HT1080 Cells on Density Gradients

Methods: Holo-transferrin was radioiodinated with ^{125}I-Na (NEN) and Iodi-Beads (Pierce) as described previously (43). Subcellular fractionation was performed as described previously (43). In brief, HT1080 cells were incubated with radioiodinated holo-transferrin (^{125}I-Tf) either at 4°C for 1 h or sequentially at 4°C and at 18°C for 1 h each. In the latter experiment, remaining ^{125}I-Tf was released from cell surface by acid wash. Incubation at 18°C produces a block in transfer of endocytosed molecules from early to late endosomes and leads to their accumulation in early endosomes (43). Postnuclear supernatants were fractionated on Percoll density gradients, and fractions were processed for direct gamma counting of ^{125}I-Tf. In some cases, after binding of ^{125}I-Tf at 4°C cells were subjected to hypotonic shock by incubation in hypotonic buffer that was depleted of potassium. This treatment has been documented to selectively block clathrin-coated pit mediated endocytosis (44). Sequentially, these cells were also incubated for 1 h at 18°C to allow uptake of ^{125}I-Tf followed by acid wash to release the not internalized ^{125}I-Tf from the cell surface.

We have performed similar experiments to investigate the endocytic pathway of megalin using L2 rat yolk sac cells (43). The protocol described here was used to determine where plasma membrane-derived vesicles and the early endosomal compartment sediment in the gradient.

Results (see Figure 8): After 4°C binding, ^{125}I-Tf sedimented in gradient fractions $^{\#}$4-6 (plasma membrane) whereas after subsequent incubation at 4°C and 18°C the internalized ^{125}I-Tf sedimented in fractions $^{\#}$8-10 (early endosomal compartment). Hypotonic shock in conjunction with potassium depletion blocked endocytosis of ^{125}I-Tf at 18°C completely. The radioactivity detected in fraction #1 represents material which did not enter the gradient.

Conclusion: Our experiments have indicated that this procedure is applicable to HT1080 cells as endosomes could be separated from the plasma membrane on Percoll gradients based on binding and uptake of ^{125}I-Tf. Experimental procedure to block the internalization via the clathrin-coated pit dependent pathway was successfully used on HT1080 cells.

We also examined the binding properties of FITC-conjugated uPA on Hs578T cells to begin our studies investigating the endocytic fate of uPA and uPAR in breast cancer cells (for more detail see Methods and Results to Figures #12 and #13 in Dr. Farquhar's Progress Report #DAMD-96-1-6317). In brief, we found that FITC-uPA binds to the cell surface and in some cases localizes to areas that are characteristic of focal contacts. The binding of FITC-uPA was specific as it was inhibited by the AE78 peptide. After uptake of FITC-uPA during the 18°C temperature block, we found uPA colocalized with Texas Red labeled transferrin suggesting it was accumulated in early endosomes.

3. Clathrin Coated Pit Mediated Uptake of uPAR in HT1080 cells

Methods: HT1080 cells were acid-washed in glycin buffer, pH 3, at 4°C for 3 min to release any endogenous bound uPA from uPAR. Cell surface proteins were biotinylated with

400μM Biotin XX (Molecular Probes) at 4°C for 35 min. After neutralization of biotin with 20 mM glycine for 15 min, cells were incubated with uPA:PAI-1 complexes at 4°C or sequentially at 4°C and 18°C for 1 h each. The latter was performed with and without hypotonic shock and potassium depletion. Complexes were prepared as described previously (45). In addition, the 4°C/18°C experiments were performed using conditioned medium from HT1080 as a rich source of endogenous uPA:PAI-1 complexes (46) to induce internalization of uPAR. Postnuclear supernatants were fractionated as described in Methods for Figure #8 and processed for immunoprecipitation (43) of biotinylated uPAR using anti-uPAR MAb. Precipitated proteins were further processed and separated by SDS-PAGE, transferred onto PVDF membranes, blotted with streptavidin coupled to horseradish peroxidase (HRP) for 15 min at room temperature. Bound streptavidin was visualized by chemiluminescence detection.

Results (see Figure 9): A. At 4°C biotinylated uPAR is found primarily in fractions #4-6 where it co-sediments with plasma membrane bound transferrin (see Figure #8). **B.** After internalization at 18°C, biotinylated uPAR was found in fractions #8-10, co-sedimenting with Tf in endosomes. This shift into the denser gradient fractions was completely blocked after hypotonic shock in combination with potassium depletion (**C**) suggesting internalization is mediated by clathrin-coated vesicles. Incubation with HT1080-conditioned medium (**D**) could mimic the effect of pre-formed uPA:PAI-1 complexes by inducing a shift of biotinylated uPAR into the denser fractions of the gradient.

These results demonstrate that uPAR with bound uPA:PAI-1 complexes are taken up via clathrin-coated pits and delivered to early endosomes.

Currently, we investigate this uptake of uPAR in greater detail. We will use RAP, a potential blocker of ligand binding to LRP, to block uptake of the complexes and of uPAR. In addition, we will attempt to immunoisolate early endosomal vesicles from gradient fractions #8-10 using either anti-LRP_{ct} (human) or anti-TfR_{ct} (human) MAb. Those isolated vesicles will be further studied for their content of biotinylated surface-derived uPAR and LRP, and for uPA:PAI-1 complexes.

Summary of New Findings: During the course of our investigations as to the fate of uPA, PAI-1, uPAR, and scavenger receptors at the cell surface, we have identified two important abnormalities in the highly tumorigenic MDA-MB-231 cells: 1) there is a defect in recycling of LRP after endocytosis which may contribute to the abnormal extracellular accumulation of uPA and PAI-1 in these cells, and 2) they express a truncated form of uPAR which may have significant functional implications for uPA proteolytic activities at the cell surface. We have also shown that LRP and uPAR are present at the cell surface in a complex that can be coprecipitated, and that uPAR is internalized into endosomes via clathrin-coated vesicles. We have now begun to explore in greater depth the interactions between scavenger receptors, uPAR, and uPA:PAI-1 complexes on the cell surface, and their endocytic trafficking and clearance. Our next goal will be to determine whether there are differences between different cell lines in the interplay between the elements of the complex in their cell surface distribution and uptake.

IV. OVERALL CONCLUSIONS

General Statement: In the first year of this fellowship significant progress was made in characterizing the expression levels and subcellular distribution of the scavenger receptors (LRP and megalin), uPAR, uPA, and PAI-1 in 3 breast cancer cell lines (MDA-MB-231, Hs578T, and MCF-7 cells) and 1 normal mammary epithelial cell line (184-B5). We also made progress on each of the tasks outlined in the Statement of Work for the first year and have moved ahead of the original schedule in some cases (e.g., quantitative analysis of scavenger receptors). The most important general conclusion we could make at that point was that we had documented a

number of differences between the cell lines studied in either expression levels of these proteins or their localization supporting our original working hypothesis --i.e., that scavenger receptors may be abnormal in breast cancer cells and that they are the missing link that needs to be thoroughly investigated in order to fully understand the connection between the high concentrations of uPAR, uPA and PAI-1 in mammary tumors and malignancies in breast cancer.

In the second year of our funding we made significant progress on Specific Aims #1, #3 and #4. We have obtained evidence that LRP and uPAR are localized together at the cell surface in several cell types and can be coprecipitated. We have also obtained evidence that uPAR and uPA are taken up into the cell by endocytosis via clathrin-coated vesicles. We have documented several abnormalities in the MDA-MB-231 cells--ie., they have a defect in recycling of LRP and they express a truncated form of uPAR. Again we have made progress on each of the tasks outlined in the Statement of Work.

Highlights of our results and conclusions to date are as follows.

1. The scavenger receptors, LRP and megalin, are expressed in all tumor cell lines investigated except MDA-468 cells which do not express detectable LRP. In the case of LRP, these receptors function normally in ligand binding. Megalin is expressed in all 3 breast cancer cell lines as well as the normal cell line by immunoblotting. The discovery of a lack of LRP expression in MDA-MB-468 cells provides us with an opportunity to study the role of megalin in uPAR uptake devoid of interference with LRP.

2. There is a direct correlation between the level of LRP expression in breast cancer cell lines and tumorigenicity in nude mice in the cell lines studied so far. Quantitative analysis of LRP expression levels in the most malignant cell line studied (MDA-MB-231), by immunoblotting, immunoprecipitation, and ligand binding demonstrates that they express 10X greater levels of LRP than non tumor-producing breast cancer cells (MCF-7) or a normal mammary cell line (184-B5). Ligand binding and uptake studies suggest that although ligand binding to LRP is normal in these cells, receptor recycling is impaired in this cell type.

3. RAP is expressed at similar levels in both normal and tumor cell lines and is found in its usual location in the ER. Thus the distribution and expression of RAP are similar to other cell types of varied origin studied previously. From these data it is safe to conclude that the differences in the expression of scavenger receptors cannot be explained by differences in the level of expression of their chaperone, RAP.

4. The levels of expression of uPAR at the cell surface are considerably higher in the two more aggressive (estrogen-insensitive) breast cancer cell lines as compared to the estrogen-sensitive and normal cell line. This matches the finding that expression of uPAR is higher in more malignant breast tumors with increased potential for metastasis and indicates that the cultured cell lines provide a valid *in vitro* model to study the role of uPAR in breast cancer. Moreover, our studies to date suggest that occupied uPAR may have a different distribution in the more malignant cell lines. Unoccupied uPAR are seen along the entire cell surface in all cell lines, but in MDA-MB-231 and Hs578T cells, occupied uPAR were also associated with focal adhesion sites on the basal cell surface. This suggests that uPAR could play a significant role in localizing uPA proteolytic activity near focal contacts and thereby promote cell detachment and migration. We need to verify this finding by colocalization of uPAR at the EM level with uPA and focal adhesion markers such as integrin receptors, vinculin, paxillin and focal adhesion kinase

5. The protease, uPA, is found at the cell surface in all four cell lines. However, in the two estrogen-insensitive cell lines but not in normal cells, uPA is also found at focal adhesion sites. These results suggest that increased uPA proteolytic activity may exist at sites of cell

adhesion which would greatly enhance cell detachment and migration of breast cancer cells as compared to normal cells.

6. PAI-1 is a secreted protein that is known to associate with extracellular matrix (ECM) proteins. Our immunocytochemical results to date show that 1) PAI-1 is expressed in both estrogen insensitive breast cancer cell lines and is deposited in the ECM, and 2) it is not found in the ECM of the estrogen sensitive or normal cells. These results suggest that the expression of PAI-1 may directly correlate with the invasive phenotype of the most aggressive breast cancer cell lines. We will perform quantitative studies to determine if any PAI-1 is being made by 184-B5 cells and other normal mammary cell lines and further evaluate PAI-1 expression levels in the breast cancer cells.

7. uPAR and LRP were found to be colocalized at the cell surface and could be coprecipitated indicating their assembly into a complex at the cell surface. Unlike many other GPI-linked proteins, uPAR was not found in caveolae.

8. Internalized uPAR with bound uPA colocalizes with transferrin and cosediments with transferrin-containing vesicles indicating that the complex is taken up by endocytosis via clathrin-coated pits in at least some cell lines.

9. Abnormalities were found in ligand degradation following LRP-mediated endocytosis in one of the tumor-derived cell lines (MDA-MB-231), and we have clearly demonstrated that this is due to a defect in LRP recycling. Moreover, we have shown that these cells have a truncated form of uPAR on their surfaces which is missing the uPA-binding Domain 1. The absence of Domain 1 may have significant impact on uPA cell surface activities and clearance in these cells.

The studies described in this progress report are currently being prepared for publication in an peer-reviewed journal: Czekay, R.-P. et al.: "Urokinase receptor, uPAR, with bound uPA:PAI-1 complexes is internalized by LRP via clathrin-coated vesicles.". In addition, these data will be presented at the 90th Annual Meeting of the American Association for Cancer Research (April 10-14, 1999) in Philadelphia, Pennsylvania.

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APPENDICES

TABLE I

Antibodies	Type	Designation	Assay			
			IG	IF	IB	IP
anti-human LRP (recognizes the cytoplasmic tail)	PAb	anti-LRP_{ct} (human)	nd	+	+	+
anti-human LRP	PAb	anti-LRP (human)	+	+	+	+
anti-human LRP (recognizes the ectodomain)	MAb*	anti-LRP_{ec} (human)	+/-	+	+/-	+
anti-rat megalin (recognizes the cytoplasmic tail)	PAb	anti-$megalin_{ct}$ (human)	nd	+	+	+
anti-rat megalin	PAb	anti-megalin (rat)	nd	+	+/-	+
anti-rat megalin (against ligand-binding domains I or II)	PAb	anti-$megalin_{LBD\ I,II}$ (rat)	nd	+	+	+
anti-human megalin	PAb*	anti-megalin (human)	nd	+	+	+
anti-human uPAR (American Diagnostica)	PAb	399 R	+	+	nd	nd
anti-human uPAR (American Diagnostica) (recognizes Domain 1, occupied receptor)	MAb	3936	+	+	nd	nd
anti-human uPAR (American Diagnostica) (recognizes Domain 1, un-occupied receptor)	MAb	3937	+	+	nd	nd
anti-human uPAR (American Diagnostica (recognizes Domain 2 of the receptor)	MAb*	3932	+	+	nd	nd
anti-human uPAR (M. Shuman, UCSF) (recognizes epitop between Domains 1 and 2)	MAb*	anti-$uPAR_{MS}$ (human)	+/-	+	+/-	+
anti-human uPA (B chain) (American Diagnostica)	MAb	394	nd	+	+	+
anti-human uPA (Calbiochem)	MAb*	anti-uPA (human)	nd	+	+	nd
anti-human PAI-1 (American Diagnostica))	MAb*	anti-PAI-1 (human)	nd	+	+	nd
anti-rat RAP	PAb	anti-RAP (rat)	nd	-	+	+
anti-human RAP	PAb	anti-RAP (human)	nd	+	+	+
anti-human transferrin receptor (recognizes the cytoplasmic tail)	MAb*	anti-TfR (human)	+	+	nd	nd
anti-human transferrin receptor (recognizes the cytoplasmic tail)	PAb*	anti-TfR_{ct} (human)	+	+	nd	nd
anti-rat caveolin	PAb*	anti-caveolin	+	+	nd	nd

+/- = of limited use
\+ = useful in the indicated assay
nd = not done
PAb = polyclonal antibody
MAb = monoclonal antibody
* = new antibodies used during second year of funding

IG = immunogold
IF = immunofluorescence
IB = immunoblotting
IP = immunoprecipitation

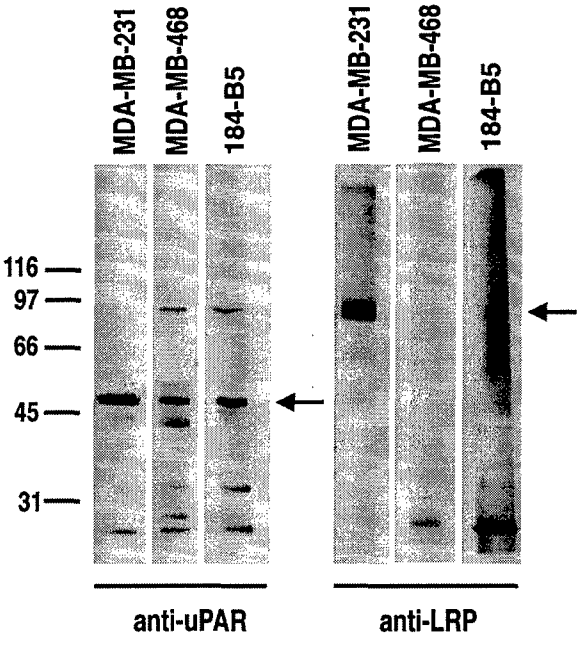

Figure 1: Expression of LRP and uPAR in estrogen-insensitive breast cancer and normal mammary epithelial cell lines.

Equal amounts of protein (50μg) from whole cell lysates were immunoblotted with anti-uPAR and anti-LRPct polyclonal antibodies. Although, uPAR was expressed by both estrogen-insensitive cell lines, LRP could not be detected in MDA-MB-468 cells.

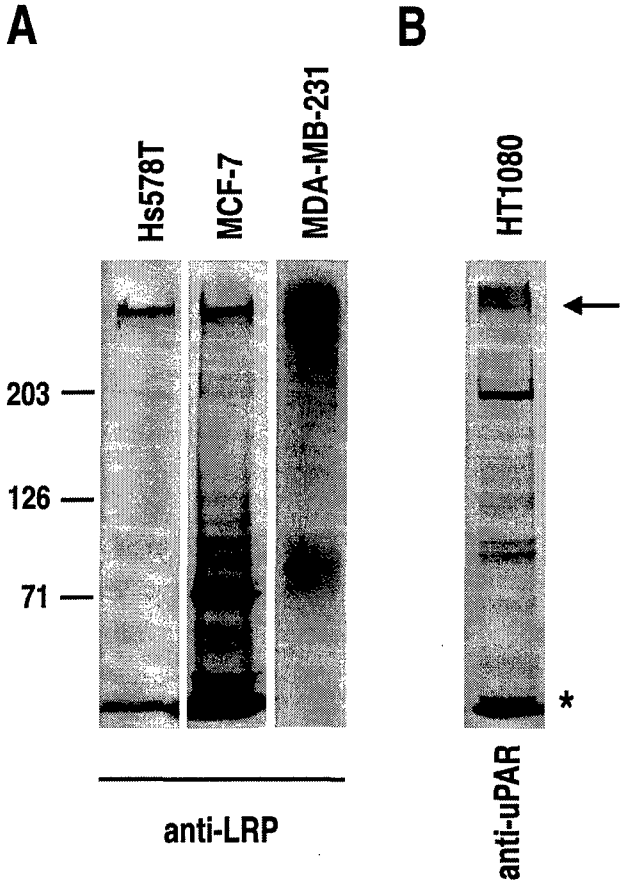

Figure 2: Cell surface expression of LRP in tumor-derived epithelial cell lines.

Radioiodinated cell surface proteins were immunoprecipitated with a newly obtained monoclonal antibody (8G1) against the ectodomain of human LRP. **A.** Surface expression of LRP was significantly the highest in estrogen-insensitive MDA-MB-231 cells. **B.** Using a new anti-uPAR monoclonal antibody we could coprecipitate uPAR and LRP from a human fibrosarcoma cell line, HT1080.

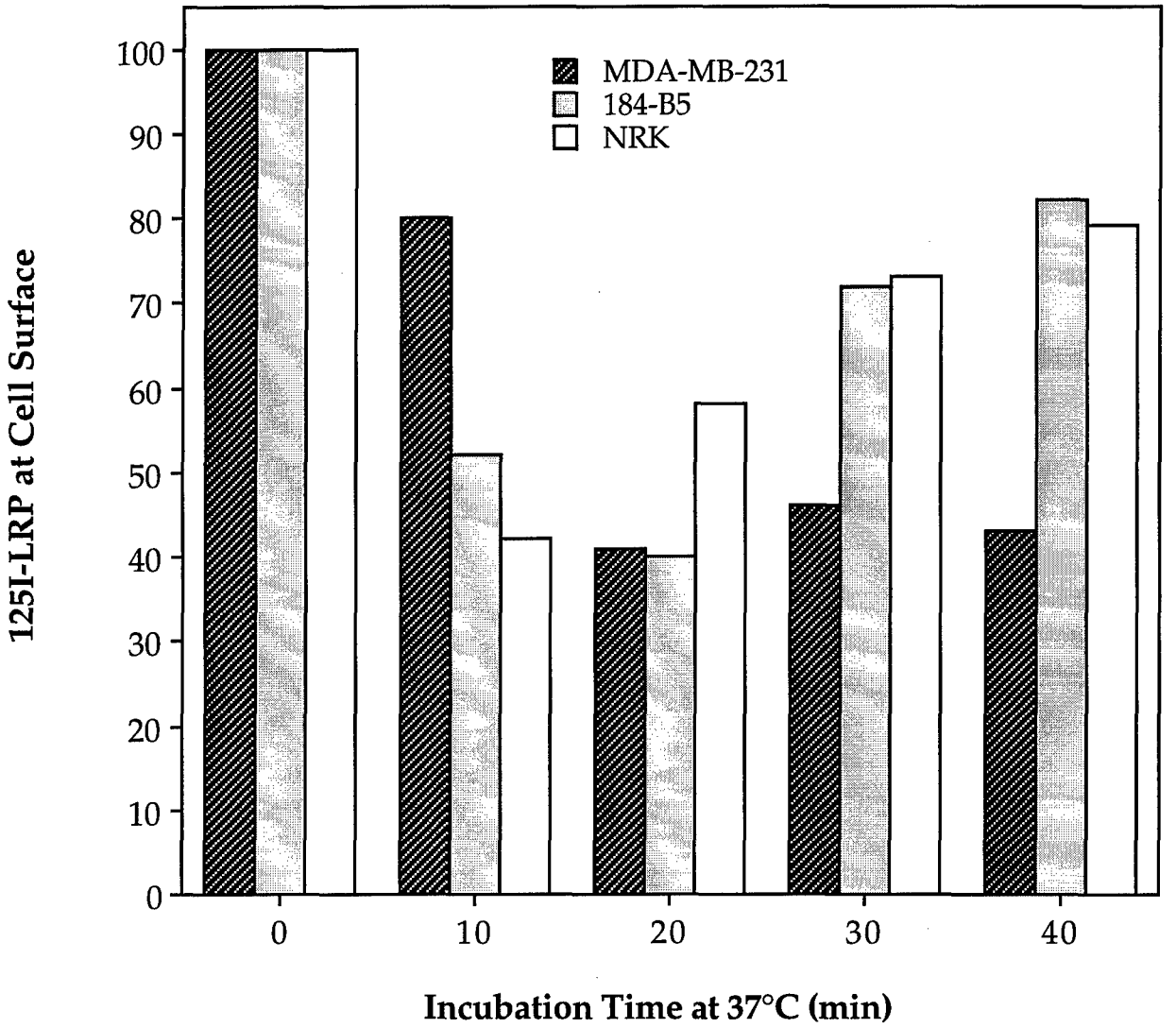

Figure 3: Recycling of LRP is blocked in MDA-MB-231 cells.

Cell surface radioiodinated cells were incubated with α2M at 4°C and subsequently incubated at 37°C for the indicated time periods. After cell surface immunoprecipitation at 4°C with anti-LRP MAb (8G1), the amount of radiolabeled LRP at the cell surface at each time point was quantitated by densitometry. Normal cell lines, 184-B5 and NRK (Normal Rat Kidney), and tumor-derived MDA-MB-231 cells show a 60% decrease in surface LRP during the first 20 min at 37°C. The controls show an 50% increase of labeled LRP at the surface after an additional 20 min at 37°C. The amount of radioiodinated LRP at the surface of MDA-MB-231 cells remained unchanged during the rest of the incubation time indicating that the cancer cells do not recycle the receptor to the cell surface.

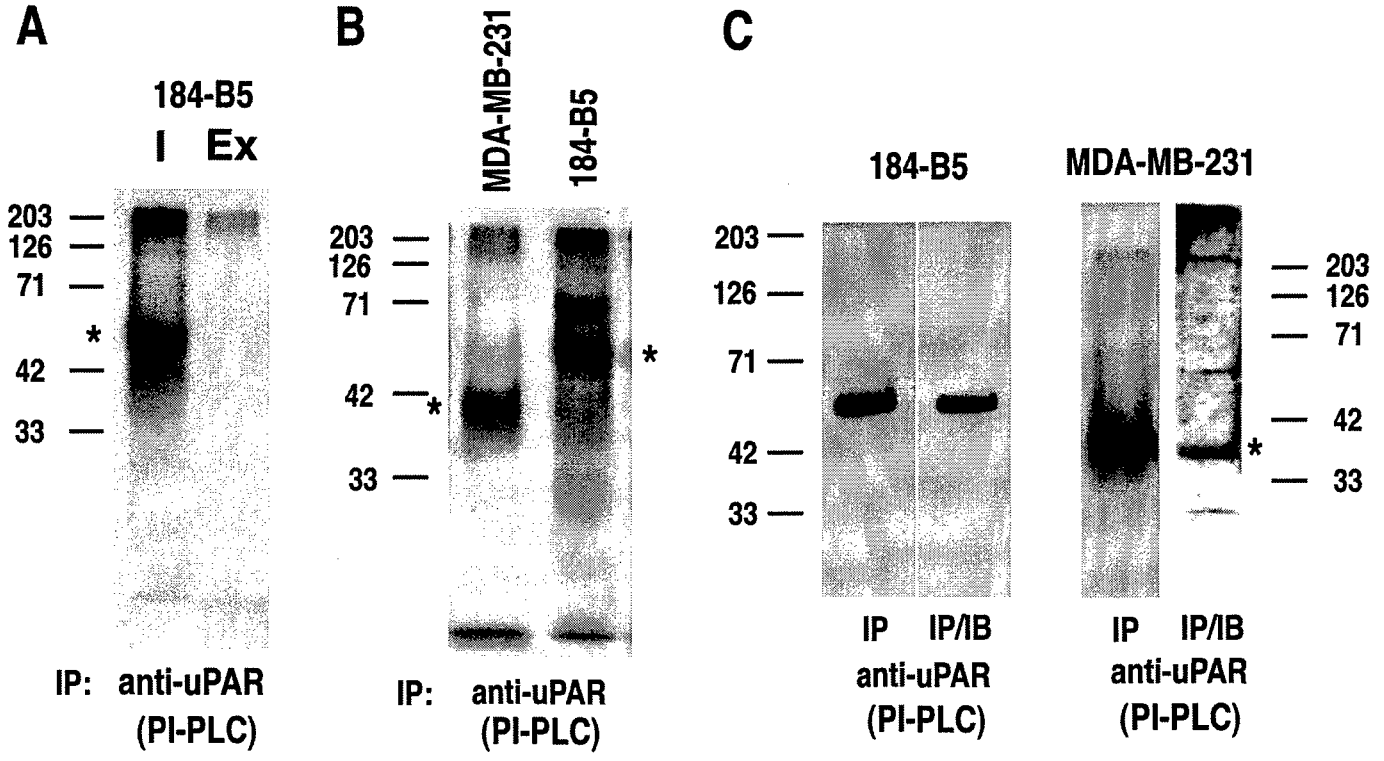

Figure 4: The breast cancer cell line MDA-MB-231 expresses a smaller form of uPAR at the cell surface than normal mammary epithelial cells, 184-B5.

Radioiodinated uPAR was cleaved from the cell surface during incubation with phosphoinositol-specific phospholipase C (PI-PLC). uPAR was immunoprecipitated with anti-uPAR MAb from the PI-PLC-incubation buffer (I) and detergent extracted proteins (Ex). **A.** The surface pool of uPAR is completely released from the cell surface into the incubation buffer (I) by PI-PLC. **B.** Compared to normal cells, the precipitated protein released from MDA-MB-231 cells shows a significantly higher mobility by SDS-PAGE. **C.** Both precipitated proteins were confirmed as uPAR by sequential immunoblotting.

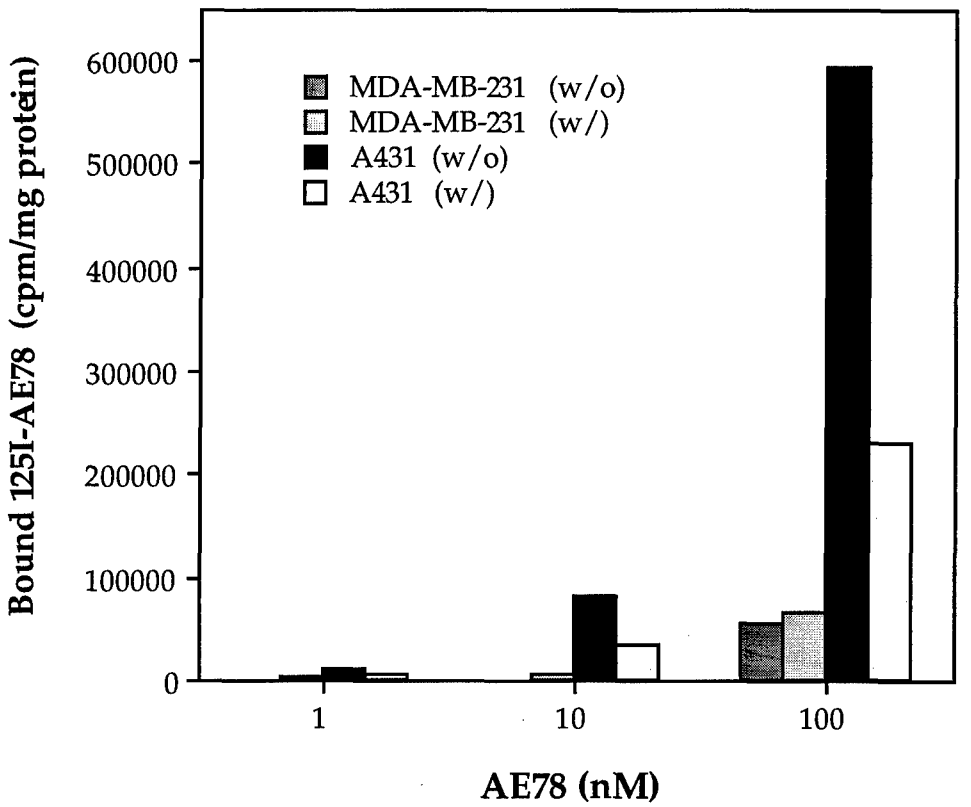

Figure 5: The uPA-derived peptide, AE78, does not bind to uPAR on cultured MDA-MB-231 cells.

MDA-MB-231 cells and a human epidermoid cancer cell line, A431, were incubated with varying concentrations of radioiodinated AE78 at 4°C (w/o). In parallel experiments binding was competed with 200-fold excess of unlabeled peptide (w/). Amounts of bound radiolabeled peptide were determined. A431 cells, which express high amounts of full-length functional uPAR at their surface, showed a 60% competition of total binding of AE78, whereas no specific binding of the uPA-specific peptide could be detected in MDA-MB-231 cells.

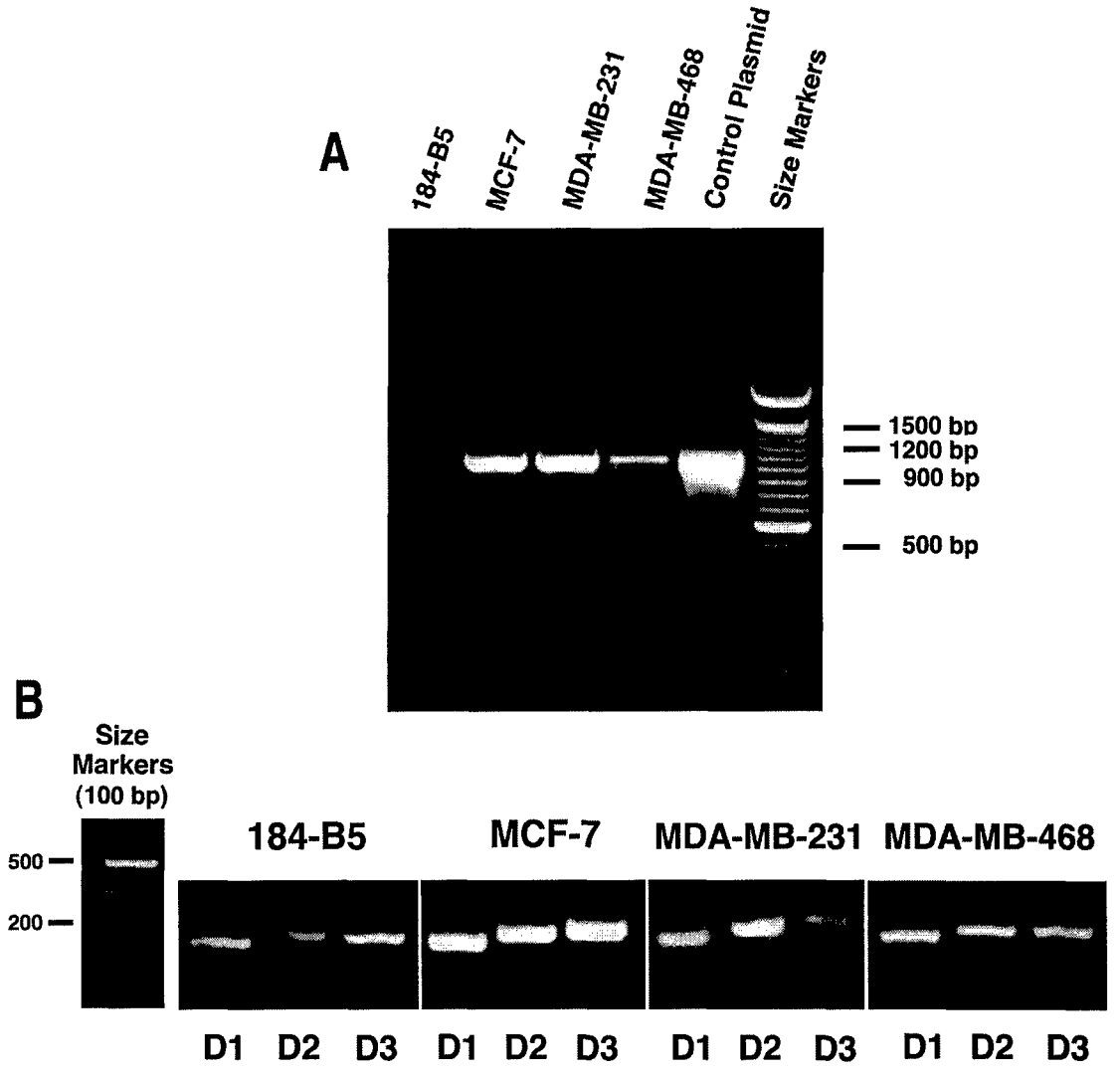

Figure 6: Normal mammary epithelial cells and three human breast cancer cell lines express messenger-RNA (mRNA) for full-length uPAR encoding all three functional domains (D1, D2, and D3) of the mature receptor. Encoding regions were amplified from total RNA using RT-PCR as described in detail in Methods. A control plasmid encoding human uPAR was obtained from the Human Genome Project. **A.** All tested cell lines show mRNA for full-length uPAR. **B.** All these mRNAs all contain the information for the three functional domains of uPAR, D1-D3.

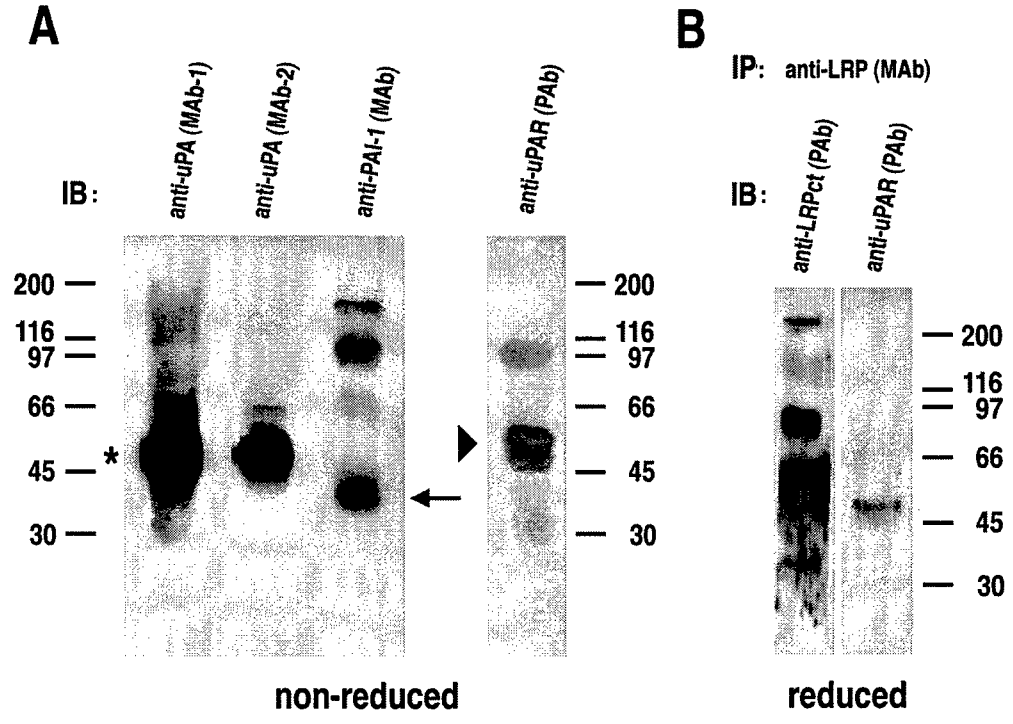

Figure 7: uPAR on the surface of HT1080 cells can be co-immunoprecipitated with LRP using anti-LRP monoclonal antibodies (MAb).

Whole cell lysates were processed for immunoblotting with antibodies against uPA (MAb-1, MAb-2), PAI-1, uPAR (A) or for immunoprecipitation with anti-LRP MAb (8G1) followed by IB with anti-LRP_{ct} or anti-uPAR PAb (B). **A.** Immunoblotting confirmed the expression of uPA, PAI-1 and uPAR in HT1080 cells. **B.** Immunoblot with anti-uPAR antibodies confirmed the coprecipitation of uPAR and LRP from the cell surface of HT1080 cells with anti-LRP MAb.

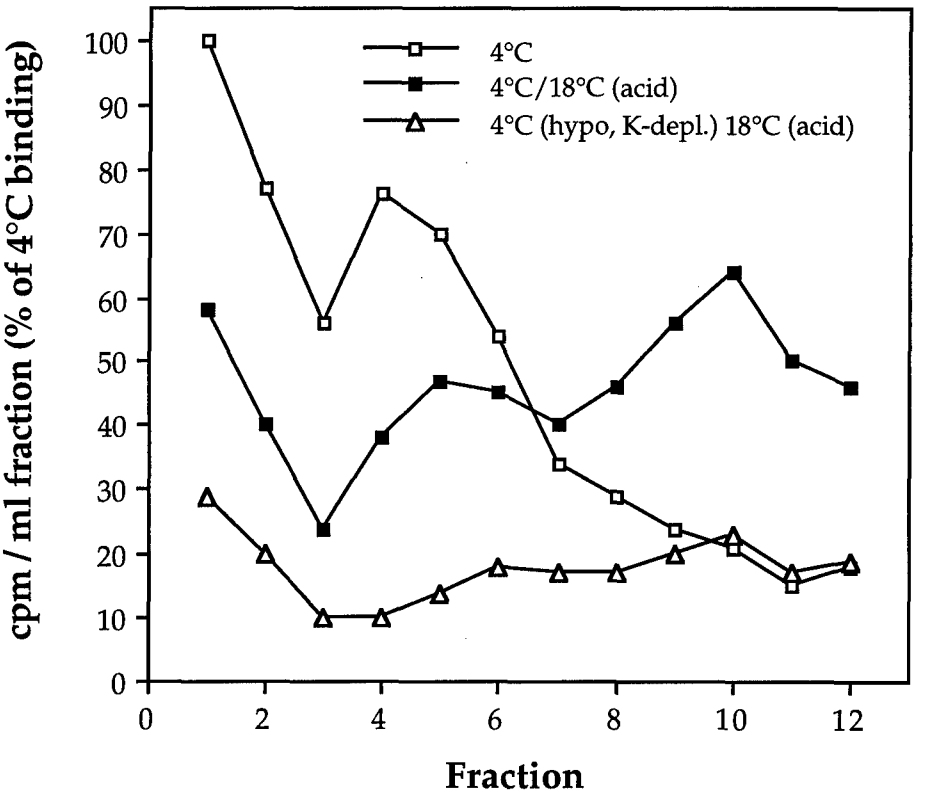

Figure 8: Uptake of transferrin by HT1080 cells is blocked by hypotonic $K^{(+)}$-depletion.

HT1080 cells were incubated with radioiodinated transferrin (^{125}I-Tf) as described in Methods. Postnuclear supernatants were fractionated on Percoll density gradients, and fractions were processed for direct gamma counting of ^{125}I-Tf. Data show that plasma membrane bound ^{125}I-Tf (4°C) sediments in fractions #4-6 and after incubation at 18°C during which it is internalized into early endosomes (4°C/18°C) sedimentation of ^{125}I-Tf significantly shifts into the denser gradient fractions #8-10. Hypotonic potassium depletion, known to selectively block clathrin-coated pit dependent endocytosis, blocks uptake of ^{125}I-Tf at 18°C (4°C/hypo, K-depl./18°C) completely.

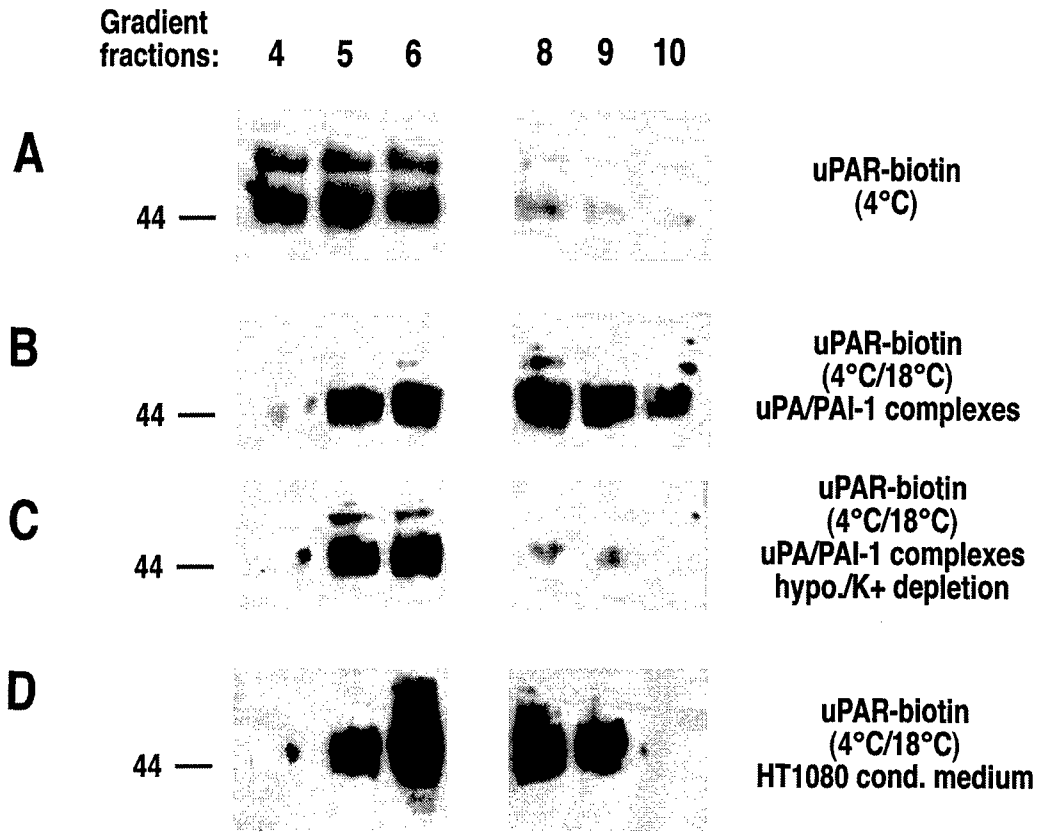

Figure 9: Surface located uPAR is internalized via clathrin-coated pits.

After release of endogenous uPA from surface expressed uPAR, cells were biotinylated at 4°C and incubated either with preformed uPA:PAI-1 complexes or conditioned culture medium from HT1080 cells. In one experiment, cells were sequentially incubated in potassium depleted hypotonic buffer to specifically block clathrin-coated pit dependent endocytosis. Cells were exposed to 18°C for 1 h and postnuclear supernatants were fractionated on Percoll density gradients and processed as described in Methods. **A.** Biotinylated surface uPAR sediments in gradient fractions #4-6. **B.** After binding of uPA:PAI-1 complexes and incubation at 18°C the majority of this surface pool is internalized. Biotinylated uPAR is now detectable in fractions #8-10. **C.** Hypotonic shock and potassium depletion can block this internalization completely. **D.** Incubation with HT1080 conditioned medium as a rich source of endogenous uPA:PAI-1 complexes also induced a shift of biotinylated surface uPAR into denser fractions #8 and #9. The result show that uPAR with bound uPA:PAI-1 complexes are taken up via clathrin-coated pits.

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